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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

II. The Influence of pH, Temperature, and Oxygen Tension on Hemolysis by Hypotonic Solutions

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I

In the first paper of the present series (Jacobs, 1930), some of the more important advantages of the erythrocyte as material for the study of cell permeability were mentioned. At the same time it was pointed out that because of its peculiar sensitiveness to certain environmental factors whose effects upon ordinary cells are much less noticeable, the erythrocyte has acquired an undeserved reputation for variability in its osmotic behavior. In the present paper, the nature and magnitude of the effects of three such factors, namely, pH, temperature and oxygen tension, will be considered. It will be shown that at least two of them have for the erythrocyte an importance entirely out of proportion to that observed in the case of other cells and that their neglect in osmotic studies on this type of material is certain to lead to serious difficulties.

A survey of the literature shows that the importance of the first of the three factors was recognized by Hamburger, the pioneer worker in the field of osmotic hemolysis, whose numerous earlier studies are conveniently summarized in his book, "Osmotischer Druck und Ionenlehre" (1902). Hamburger, to be sure, did not distinguish clearly between the titratable acidity or alkalinity of a solution and its true reaction, now commonly expressed as pH; but he did, nevertheless, show conclusively that changes of the blood in the acid direction, whether

produced by carbon dioxide or by other acids, result in a lowered osmotic resistance of the erythrocytes and that changes in the alkaline direction have the opposite effect. He correctly associated these changes in resistance with the volume changes, observed by von Limbeck (1895), which occur under the same conditions. The volume changes he interpreted in turn as being primarily of an osmotic nature, resulting from changes in the amounts of base bound by the cell proteins and from a certain type of ionic interchange between the cell and its surroundings (1902, pages 307 and 335). In its details, Hamburger's theory was rather indefinite and in several respects it has proved to be entirely erroneous, but it does nevertheless foreshadow to some extent the most modern views on the subject.

Among workers who followed Hamburger, there was for a time a tendency to abandon the osmotic explanation of the effects of pH changes and to attribute these effects to more or less vague colloidal phenomena. Haffner (1920), for example, in a study in which actual pH values are mentioned (for most of the values in question see Jodlbauer and Haffner, 1920), and which in this respect represents an advance over the work of Hamburger, concludes that, "Da es sehr unwahrscheinlich erscheint, dass der osmotische Druck der im Zellinnern in wahrer Lösung befindlichen Substanzen durch Verschiebung der H-Konzentration erhebliche, die obigen Befunde erklärende Änderungen erfährt, so folgt, dass für das Quellungsverhalten der Zelle als Ganzes der Quellungszustand—also der Ladungszustand—gewisser Zellkolloide von ausschlaggebender Bedeutung ist." Similar views have been expressed by other workers.

More recently the osmotic theory of the effect of pH on the volume of erythrocytes (from which its application to problems of hemolysis is an easy step) has again been brought forward, this time in a far more definite and satisfactory form than previously, in the very important papers of E. J. Warburg (1922) and of Van Slyke, Wu, and McLean (1923). For a somewhat briefer discussion of the theory in question Van Slyke (1924, 1926) may be consulted. Not only did this theory provide a plausible theoretical explanation of the known facts but the observed and predicted magnitudes of the effects in question, of which that on cell volume is the one which concerns us here, were found to be in good quantitative agreement. So generally satisfactory has this theory proved to be that it is most surprising that so little account of it has as yet been taken by persons interested primarily in problems of hemolysis. One of the purposes of the present paper is to point out its general applicability to such problems.

The second of the three factors under consideration, temperature,

was also studied to some extent by Hamburger (1902, page 172), but with results which, probably because of the crudeness of his methods, were unsatisfactory. He found almost no measurable differences in the degrees of hemolysis produced by hypotonic solutions at 0°, 14° and 34° C. Such slight differences as he obtained seemed to indicate, if anything, slightly more hemolysis at higher than at lower temperatures, a result in the opposite direction from that observed when more accurate methods are employed.

A much more satisfactory piece of work is that of Jarisch (1921), in which a very clear and regular decrease in degree of hemolysis with rising temperature was obtained for a number of species of mammals. Neither the theory offered by Jarisch to account for this effect, however, nor the evidence upon which he based it are very convincing, and it seems much more plausible to look for an explanation along the same lines as those already shown to be useful in the case of pH effects. While the indirect osmotic influence of temperature upon the volume of the erythrocyte has apparently received only incidental attention, it is in all but its details included in the general theory of such volume changes.

Concerning the effect of the third factor, oxygen, on hemolysis, little information is available. Ordinary "fragility" studies are of very questionable value for reasons which will be discussed later. Strangely enough, Hamburger, whose own work had done so much to establish the importance of carbon dioxide as a factor capable of influencing hypotonic hemolysis, at times appeared to attribute the increased resistance of the erythrocytes from defibrinated blood solely to an increase of oxygen (for example, 1902, page 172), seemingly forgetting the simultaneous loss of carbon dioxide. It is obvious that conclusions of value can be drawn from such experiments only if the effects of the different variables are properly separated, and this appears not to have been the case in the work so far published in this field. On the other hand, the effects of oxygenation of the hemoglobin upon cell volume have been adequately dealt with by Van Slyke, Wu and McLean (1923) and by Henderson, Bock, Field, and Stoddard (1924), and the application of the results of these workers to problems of hemolysis is obvious.

Summarizing our present knowledge of the subject, it may be said that from the experimental point of view the general effects of pH and temperature upon the degree of osmotic hemolysis are known, at least qualitatively, even though they are only too frequently neglected in practice. The effect of oxygen tension is known with less certainty. On the other hand, the effects of all of these factors upon cell volume

can be predicted theoretically from the known properties of the erythrocyte, of which the base-binding power of its hemoglobin is one of the most important; and the calculated and observed volumes have been shown in the case of pH to be in good agreement (Warburg, 1922; Van Slyke, 1924). It remains to determine how far the simple and definite theory of osmotic volume changes is capable of accounting for the effects upon hemolysis of the three factors in question and how far less precise theories of "colloidal behavior" must be considered. Only to the extent that the first type of explanation can be shown experimentally to be inadequate will it be necessary to invoke the second.

II

Before the presentation of the experimental results, it may be pointed out that the method of hemolysis, when properly employed, is perhaps the most delicate method at present known for investigating the osmotic volume changes of cells. This highly desirable characteristic of the method is, rather paradoxically, due to a property of the biological material with which it is employed which is commonly thought of only as a disadvantage, namely,—its variability. As is well known, the erythrocytes in a given sample of blood form a highly heterogeneous population with respect to their resistance to osmotic swelling. In Fig. 1 is represented the distribution of cells of different resistances in a

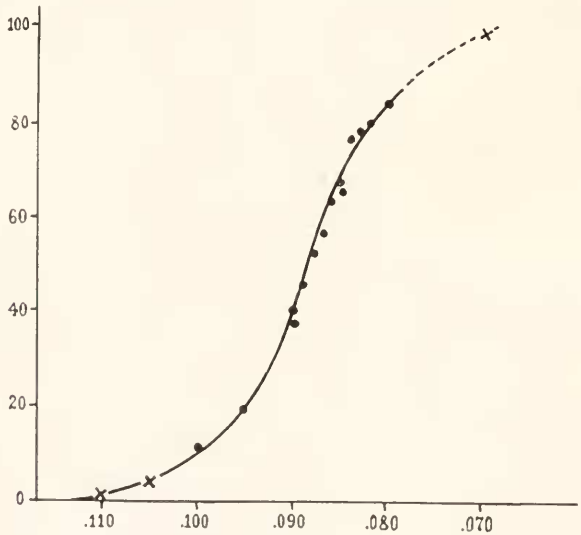

Fig. 1

Fig. 1. Variation in osmotic resistance of erythrocytes of the ox. Ordinates represent percentages of hemolysis; abscissæ, times in minutes. Conditions of the experiment as mentioned in the text.

sample of ox blood. The data were obtained under the standard conditions of 20° C., pH 7.4, the oxygen tension of room air, an exposure to the solutions for an hour with stirring and the use of such small quantities of blood that the composition of the solution does not undergo any appreciable change during the course of hemolysis. The points represented by circles, which were obtained by the method previously described (Jacobs, 1930), and those represented by the two lower crosses, which were obtained by the other author by a new method to be described elsewhere, fall very satisfactorily upon the same smooth curve. The uppermost cross is based upon a direct microscopic examination of the suspension.

The cause of the osmotic variability of the erythrocytes is not definitely known. It may be associated with differences in the properties of the membranes of different cells, either in resisting stretching or in becoming permeable to hemoglobin when stretched. Another possibility was suggested by Hamburger, namely, that different cells contain different amounts of substances such as the materials of the stroma and hemoglobin which occupy a part of the cell volume without themselves undergoing osmotic volume changes. The higher the percentage of such substances the less the swelling of the cell as a whole in a given hypotonic solution would be and the less, therefore, its tendency to undergo hemolysis.

Whatever factor or factors may be responsible for the osmotic variability of the erythrocytes, the important fact is that these cells are so numerous (approximately 250,000,000 in an ordinary drop of blood) that, on the one hand, successive samples from the same lot of blood are almost identical and, on the other, the variation is practically continuous, *i.e.*, there are represented an almost infinite number of degrees of osmotic resistance. Under these conditions, the variability of the cells instead of being a source of error becomes an advantage by rendering it possible to compare with a high degree of accuracy the osmotic properties of solutions whose concentrations differ only very slightly.

A fair idea of the degree of accuracy obtainable with the method of osmotic hemolysis without the employment of any unusual precautions is illustrated in Fig. 2. In this figure smooth curves have been drawn through the points transferred from the original kymograph record (see Jacobs, 1930). The slightly buffered NaCl solutions employed differed in concentration by 0.001 M. With one or two slight discrepancies the curves form a very regular series. It would obviously be possible in the most sensitive part of the range of the instrument without further precautions than those here taken to distinguish the osmotic effects of solutions differing from one another by 0.0002 M or less, *e.g.*, the effects

of 0.0880 M and 0.0882 M NaCl. Such a difference in concentration would produce in an osmotically perfect system a volume change of less than 0.25 per cent and in an erythrocyte in which the volume of the non-liquid part of the cell has been estimated at from 35 or 40 per cent (Ege, 1921) to 65 or 70 per cent (Gough, 1924; Krevisky, 1930) a visible change for the cell as a whole of only between 0.1 and 0.2 per cent. Differences of this order of magnitude, which are by

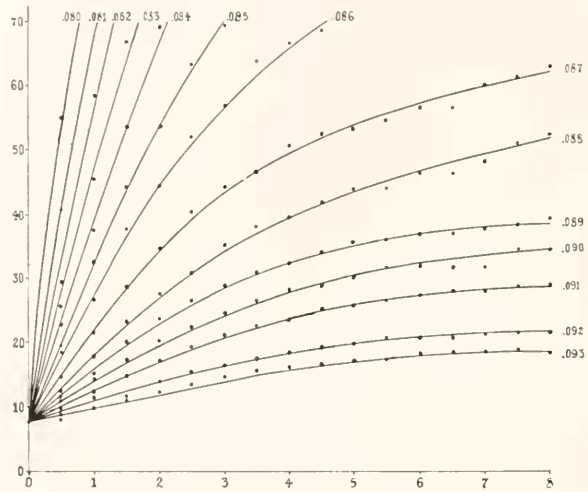

FIG. 2

FIG. 2. Effect of concentration on the rate of hemolysis of ox erythrocytes at 20° C. and pH 7.4. Ordinates represent scale readings of the instrument and abscissæ times in minutes.

no means the smallest that could with care be measured, could probably not be dealt with successfully by any other method. Even the large spherical *Arbacia* egg, the most favorable cell in certain respects for osmotic studies which has yet been employed, does not permit the accurate measurement of volume changes ten times as great.

It follows that the hemolysis method under favorable conditions is one of great delicacy. As with other delicate methods, however, the effects of disturbing factors are correspondingly serious. Some of these factors will now be considered.

III

The most important factor theoretically and the most difficult one to control practically is the pH of the medium. It is safe to say that more errors have been caused in studies of osmotic hemolysis by neglect

of this factor than of any other. Where a relatively large quantity of blood is used with an unbuffered salt solution, the resulting pH will be determined chiefly by that of the blood, which in turn will vary greatly according to the amount of CO_2 which has been allowed to escape, etc. If very small quantities of blood are employed the case is no better, since now the final pH will be more strongly influenced by that of the solutions used, which because of absorption of carbon dioxide from the laboratory air, etc. may be expected, in the absence of buffers, to vary considerably. Under such conditions, even a breath from the experimenter at the wrong time may completely ruin an experiment. It is evident, therefore, that to obtain accurate results it is important on the one hand to use very small quantities of blood and on the other to work with buffered solutions of known pH. These precautions have been used throughout this work.

In carrying out experiments upon the effects of pH changes in which concentration differences of 0.0002 M are significant, the usual phosphate buffers are not entirely satisfactory. Solutions of Na_2HPO_4 have considerably higher osmotic pressures than those of NaH_2PO_4 of equal concentration, and mixtures of such solutions in different proportions therefore involve an important variable in addition to pH. Theoretically, it is possible to find a concentration of HCl which when mixed with a solution of Na_2HPO_4 will cause no appreciable change in osmotic pressure, the resulting volume changes just balancing those in dissociation; practically, however, it is difficult to be certain that pH standards made up in this way are exactly equivalent, since the ordinary freezing point method for osmotic pressure determinations is not sufficiently delicate for the necessary tests. It has proved better in practice, therefore, to use a solution containing a fixed amount of $NaHCO_3$ and to vary the pH by adding different amounts of CO_2, a substance which not only causes no appreciable volume changes in the solution itself but which distributes itself so rapidly between the solution and the cells that its direct osmotic effect upon the latter can be considered to be zero.

The disadvantages of the bicarbonate-CO_2 buffer system are, first, that because of the volatility of the CO_2 the experiments must be carried out in tightly stoppered bottles in which stirring is somewhat difficult to accomplish and from which samples cannot be removed at will; and, second, that the highest CO_2 tensions involve a simultaneous decrease in oxygen tension which in itself has indirect osmotic effects. As will be shown later, however, these effects are small in magnitude and, on the whole, the advantages of the $NaHCO_3$–CO_2 buffer system greatly outweigh its disadvantages for experiments such as the one about to be described.

The procedure employed in this experiment was as follows. A mixture of M/1 NaCl and M/1 $NaHCO_3$ (4:1) was diluted to 0.12M, 0.11 M, etc., these concentrations referring to the two salts taken together. It is permissible to combine the concentrations of the salts in question because of their great osmotic similarity. Taking first any chosen concentration, a small part of the solution was almost saturated with CO_2. This saturated solution was then mixed with the unsaturated solution in different proportions. For most of the mixtures the amount of CO_2-containing solution was so small relatively that the oxygen tension of the resulting combination was not greatly changed; in the solutions of lowest pH, however, it was considerably reduced. This reduction might have been largely prevented by the use of pure oxygen in conjunction with the carbon dioxide, but in view of the comparatively slight osmotic effects of even fairly large variations in oxygen tension (see Section V), this refinement was not considered necessary.

As each solution was prepared, two carefully measured drops of defibrinated blood were introduced into 50 cc. of it in a closed vessel. As soon as thorough mixing had been accomplished a 30 cc. glass-stoppered bottle was quickly filled with the suspension of erythrocytes through a glass tube without unnecessary exposure to the air. The bottle, completely filled and stoppered, was placed in a water-bath at 20° C. and rocked gently for one hour. A small glass rod in the bottle, kept in continuous movement by the rocking of the latter, prevented the erythrocytes from settling to the bottom. Immediately after the bottle had been filled a pH determination was made upon a part of the remaining solution with a quinhydrone electrode designed to prevent escape of CO_2. After a series of different pH values had been studied for one concentration a second concentration was similarly employed and so on until the entire range had been covered.

The results of this experiment are represented in Fig. 3 in which percentage of hemolysis is plotted against pH, and concentrations are represented by contour lines. It will be noted that starting at any given point the percentage of hemolysis may be increased either by diminishing the concentration or the pH, and decreased by changes in the reverse direction. Furthermore, it is possible, by changing the two variables in opposite directions, to secure an exact balancing of their effects. For example, by following the level of 50 per cent hemolysis from the contour line for concentration 0.110 M to that for 0.100 M it is seen that a concentration difference of 0.010 M is here equivalent to a pH difference of 0.45. Considering the figure as a whole, there is seen to be some variation in the pH-concentration relation, though the order of magnitude remains the same, being in the vicinity of 0.5 pH units

for a concentration difference of 0.010 M. A comparison will later be made between this observed order of magnitude and that predicted by the osmotic theory of pH effects.

One further point is of interest, namely, that if, for example, a concentration change from 0.110 to 0.100 is equivalent to a pH change of approximately 0.5 and if, as is the case, a concentration difference in this region of 0.0002 M or a concentration ratio of 0.9998 has a

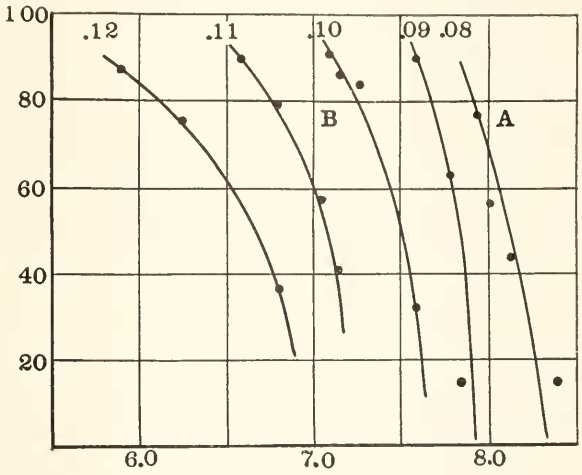

FIG. 3

FIG. 3. Effect of pH on osmotic hemolysis. Ordinates represent percentages of hemolysis; abscissæ, pH values; contour lines, concentrations in mols per liter.

visible effect upon the observed degree of hemolysis, then a pH change of 0.01 should have approximately the same effect. In other words, uncontrolled pH differences of more than this magnitude may be expected to be a source of error in experimental work.

IV

Experiments on the effects of temperature on the observed degree of hemolysis are considerably easier to carry out than those of pH because a single phosphate buffer system may be used throughout the entire series. It is true that the necessary differences in the degree of dilution of the buffer salts in such experiments will produce slight pH changes, but the effects of these changes are almost negligible in comparison with the large concentration differences involved.

In the experiment about to be described, which is a typical one chosen from several of the same general type, the erythrocytes were

those of the cat. The stock salt solution employed consisted of M/1 NaCl and M/1 Na_2HPO_4 in the proportion of 14 to 1, with the pH reduced to approximately 7.0 by means of concentrated hydrochloric acid so as to give upon dilution a pH in the vicinity of 7.4. A mixture of this sort was, of course, not exactly equivalent osmotically to M/1 NaCl, but the difference was not great and, in any case, it was relative rather than absolute concentrations which were of importance in this experiment. From the stock solution the necessary dilutions were made, treating the original solution as M/1. The pH values for the various dilutions, as determined with the quinhydrone electrode both before and after the addition of the blood, had in previous experiments been found in all cases to vary only very slightly from a value in the vicinity of 7.4. In this connection, it may be mentioned that in making progressive dilutions of the stock solution the pH changes fairly rapidly at first from its initial value of approximately 7.0, but by the time concentrations such as those here employed have been reached, at which the pH is in the neighborhood of 7.4, the effect of further dilution is slight.

The blood in the proportion of two carefully measured drops to 50 cc. was introduced into the solutions, previously prepared and brought to the desired temperatures, which in the case of those at 0° C. and 20° C. were kept constant within 0.1°, and in the case of the others

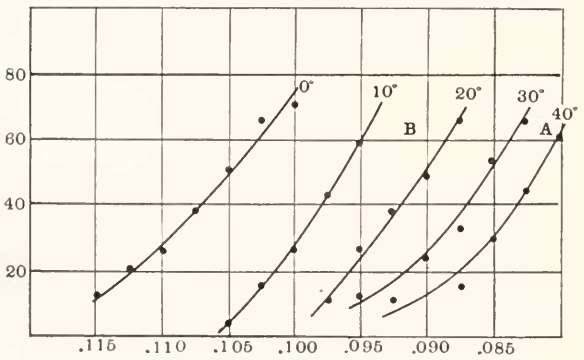

FIG. 4

FIG. 4. Effect of temperature on osmotic hemolysis. Ordinates represent percentages of hemolysis; abscissæ, concentrations in mols per liter and contour lines temperatures in ° C.

within less than 0.5° in water-baths. The solutions were gently and continuously stirred throughout the experiment. Determinations of the percentages of hemolysis attained were made at the end of one hour. This time is not sufficient for the establishment of complete equilibrium

at 0° and 10° C., but the further changes at these temperatures are slight and the disadvantages of experiments of longer duration probably outweigh their advantages.

The results of the experiment are presented in Fig. 4. The percentage of hemolysis is here plotted against concentration, and contour lines are used to represent the different temperatures. It will be observed that for the region of 50 to 75 per cent hemolysis, where the erythrocytes are presumably fairly typical and where errors in the measurements are small, a change in temperature of 10° C. is exactly balanced by one in concentration from 0.0035 M to 0.0075 M. In other words, for the region in question, a concentration difference of 0.001 M is roughly equivalent to a temperature difference of the order of magnitude of two degrees. It follows, therefore, that if a visible influence upon the observed degree of hemolysis is exerted in this region by a concentration change of 0.0002 M, the same result should be obtained by a temperature change of less than 0.5°. In the light of this relation, it is not surprising that a lack of agreement is frequently found in experiments carried out at "room temperature."

Reference may here be made to the results obtained by Jarisch (1921). From his figures on page 256 it appears that in the case of the ox, for example, a concentration of 0.473 per cent NaCl at 40° C. was equivalent to one of 0.519 per cent at 15° C. The results for several species of mammals are presented graphically on page 257 of the same paper. It should be noted, however, that the method employed by Jarisch was unsatisfactory in two respects. In the first place, only fifteen to twenty minutes were allowed for the attainment of equilibrium and, in the second place, no account was taken of anything short of complete hemolysis (as judged by the eye). The use of more refined methods would almost certainly have given a continuous fall in the curves represented on page 257 to 0° C. instead of only to 10° or 15° C. Making allowance, however, for these and possibly other differences in technique and remembering that the concentrations in the experiments of Jarisch are expressed in percentages of NaCl rather than in mols per liter, it appears that his results were of the general order of magnitude of those here reported.

V

The effects of oxygenation upon the degree of hemolysis may next be briefly described. On the whole, they prove to be much smaller in magnitude than those produced by pH and temperature changes—in fact, their practical importance as a source of error must be very small since most laboratory solutions are in equilibrium with room air, and

in such solutions hemoglobin will be almost completely oxygenated. Only if a deliberate effort were made to reduce the hemoglobin would effects of any important magnitude be expected.

Several experiments were tried at various times to determine the effects of oxygenation at constant pH upon the degree of hemolysis. The experiment here reported was a typical one. In it, ox blood was placed at 20° C. in a series of different dilutions of the buffered stock solution mentioned in Section IV. The degree of hemolysis observed

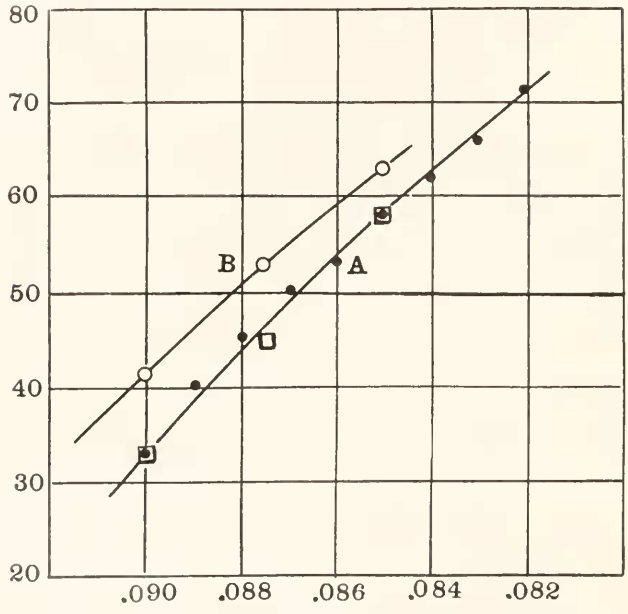

Fig. 5

Fig. 5. Effect of oxygenation on osmotic hemolysis. Ordinates represent percentages of hemolysis and abscissæ concentrations in mols per liter. The degree of oxygenation is represented as follows: open circles, complete reduction; solid circles, equilibrium with room air; squares, complete oxygenation.

at the various dilutions is represented by the solid circles in Fig. 5. After this preliminary standardization, two lots of 50 cc. each of the 0.085 M, 0.0875 M and 0.090 M dilutions were placed in separate vessels through which oxygen and hydrogen, respectively, were bubbled for ten minutes. At the end of this time two accurately measured drops of blood which had previously been introduced into the vessels but not mixed with the liquid were submerged in the oxygen-rich and oxygen-poor solutions. The gases were allowed to bubble slowly through the resulting suspensions for an hour, and then determinations of the degree

of hemolysis were made in the usual way. The pH of the various solutions did not vary beyond the limits of error of the quinhydrone electrode determinations. The hydrogen used contained approximately 0.05 per cent of oxygen, so reduction of the hemoglobin was probably almost but not quite complete.

The degrees of hemolysis obtained under the different conditions are represented in Fig. 5 by the open circles for the reduced and by squares for the oxygenated blood. From the figure it is apparent that the maximum difference obtainable under these conditions is equivalent to one in concentration of approximately 0.0016 M. Otherwise expressed, by reduction of the hemoglobin the degree of hemolysis at the concentrations in question is increased by about five to eight per cent. Neither in this experiment nor in other similar ones were significant differences observed between the effects of room air and those of pure oxygen, which is what would be expected from the known character of the oxygen dissociation curve of hemoglobin.

VI

In the three sections immediately preceding this one there have been described and measured in terms of equivalent concentration changes the effects upon osmotic hemolysis of variations in pH, temperature, and oxygen tension. It remains to determine whether the magnitude of these effects is such as can be accounted for wholly or chiefly by osmotic differences within the cell resulting from changes in the base-binding powers of the hemoglobin. Van Slyke, Wu and McLean (1923) have treated at length from this point of view the effects of pH and, to some extent, those of oxygen tension upon the related problem of cell volume, but their equations apply to the more complicated situation where the cells are suspended in a relatively small volume of a protein-containing solution (serum) whose composition varies with that of the cells. We are concerned here with the simpler case, to which these equations are not directly applicable, of cells suspended in a protein-free salt solution whose volume is so great that its composition may for practical purposes be considered to be constant. It will be necessary, therefore, following in part the method of Van Slyke, to derive an equation applicable to this type of system by which the theoretical volume changes associated with changes in the base-binding powers of the hemoglobin may be estimated.

The substances of which account must be taken in the derivation of the equation are the cations of the system (chiefly K° and Na°) together designated as B (base), and the anions exclusive of Hb' (chiefly

Cl' and HCO_3') together designated as A', hemoglobin ions Hb' and hemoglobin uncombined with base, together designated as Hb and base bound by hemoglobin, designated as BHb. The question of the degree of oxygenation of the hemoglobin need not be raised until later. Following Van Slyke, the symbols $(B)_c$, $(A)_c$, $(B)_s$, $(A)_s$, etc. will be used to refer to the amounts of B and A in the cells and solution, respectively, in a given quantity of the suspension and $[B]_c$, $[A]_c$, $[B]_s$, $[A]_s$, etc. to the corresponding *concentrations* expressed per unit amounts of water rather than per unit volume of solution, *i.e.*, $[B]_c = (B)_c/(H_2O)_c$. Van Slyke uses as his units the amounts of the substances other than water in milli-equivalents and of water in kilograms for one kilogram of blood. The resulting concentrations are therefore expressed in milli-equivalents per kilogram of water. In the present treatment, where relative results only are desired, the particular units employed are of no consequence as long as they are used consistently throughout.

With regard to the measurement of quantities and concentrations of hemoglobin, Van Slyke, in the absence of definite knowledge at the time his work was done of the molecular weight of this substance, used as an equivalent of hemoglobin the amount that combines with one mol of oxygen. This assumption leads to calculated osmotic pressures for hemoglobin which, in the light of our present knowledge, are probably too high. However, according to the equation given by Adair (1925, page 533), it would appear that the discrepancy is certainly much less than it would be if the true molecular weight of hemoglobin could be employed in the usual way for calculating the osmotic pressure of concentrated solutions. In view of this fact, and in the absence of any very definite knowledge concerning the exact contribution, which in any case is relatively small, of hemoglobin to the total osmotic pressure within the erythrocyte, we may without serious error continue to employ Van Slyke's convenient assumption, particularly in a case like the present one where comparative results only are desired.

In order that osmotic equality inside and outside of the cell may exist, which is known to be the case with the erythrocyte, whose delicate wall is incapable of supporting in either direction an excess of osmotic pressure, the following relation must hold between the concentrations of the various osmotically significant substances:

$$[B]_c + [A]_c + [Hb]_c = [B]_s + [A]_s \qquad (1)$$

or otherwise expressed,

$$\frac{(B)_c + (A)_c + (Hb)_c}{(H_2O)_c} = \frac{(B)_s + (A)_s}{(H_2O)_s}. \qquad (2)$$

Since the cell is impermeable to hemoglobin and to base but permeable to water, $(Hb)_c$ and $(B)_c$ will remain constant, while $[Hb]_c$ and $[B]_c$ will tend to vary. Indeed, the values of the latter quantities may be used as a measure of the volume of the "liquid" portion of the cell and indirectly of that of the cell as a whole, provided that information is available concerning the bulk of the "non-liquid" materials. The erythrocyte is known to be permeable to the osmotically important anions, so neither $(A)_c$ nor $[A]_c$ will be fixed but will in general vary in such a way that the Donnan ratio, $[A]_c/[A]_s$, will have the value determined by the other properties of the system. (The use of concentrations in place of activities introduces no great error; for the justification for assuming complete dissociation of the various salts involved —Van Slyke, Wu and McLean (1923)—may be consulted.)

If now in equation (2), $(B)_c - (BHb)_c$ be substituted for $(A)_c$, $2(A)_s$ be substituted for $(B)_s + (A)_s$ and the terms be suitably rearranged we obtain:

$$\frac{(H_2O)_s}{(H_2O)_c} = \frac{2(A)_s}{2(B)_c - (BHb)_c + (Hb)_c}. \tag{3}$$

Similarly, by substituting in equation (2) $(A)_c + (BHb)_c$ for $(B)_c$, $2(A)_s$ for $(B)_s + (A)_s$, and rearranging, we have:

$$\frac{2(A)_s}{(H_2O)_s} = \frac{2(A)_c}{(H_2O)_c} + \frac{(BHb)_c + (Hb)_c}{(H_2O)_c}. \tag{4}$$

Dividing both sides of equation (4) by $2(A)_s/(H_2O)_s$, substituting the value of $(H_2O)_s/(H_2O)_c$ from equation (3) and remembering the definitions of $[A]_c$ and $[A]_s$ and of the Donnan ratio, r, we have finally:

$$r = 1 - \frac{(BHb)_c + (Hb)_c}{2(B)_c - (BHb)_c + (Hb)_c}. \tag{5}$$

Up to this point Van Slyke, Wu and McLean have been followed in principle, and equation (5) is the same as their equation (14) except that $(BP)_s$, the base bound by the protein in the solution, is here zero, there being no protein present in the solution. The base bound by protein in the cell is also here designated as $(BHb)_c$ instead of as $(BP)_c$. Beyond this point a somewhat different treatment of the problem has been found convenient.

If under any given conditions of pH, temperature and oxygenation, the amount of base bound by unit amount of hemoglobin be represented by F, we may write $F(Hb)$ instead of (BHb) and equation (5) becomes:

$$r = 1 - \frac{(Hb)_c\ (1 + F)}{2(B)_c + (Hb)_c\ (1 - F)}. \tag{6}$$

Substituting in equation (1) $r[A]_s$ for $[A]_c$ and $F[Hb]_c$ for $[BHb]_c$, we obtain:

$$[Hb]_c = \frac{2[A]_s\ (1-r)}{1+F}. \tag{7}$$

Introducing into (7) the value of r from (6):

$$[Hb]_c = \frac{2[A]_s\ (Hb)_c}{2(B)_c + (Hb)_c\ (1-F)}. \tag{8}$$

As mentioned above, the concentration of hemoglobin $[Hb]$ is determined by the amount of water in some given quantity of cells, so that $1/[Hb] = kW$, where W is the water in one cell and k is a constant whose value need not be determined since it will subsequently be eliminated. Representing the ratio $(B)_c/(Hb)_c$ by R and substituting C, the concentration of the salt in the external solution, for $[A]_s$, we obtain from equation (8):

$$\frac{1}{[Hb]} = kW = \frac{2R+1-F}{2C}. \tag{9}$$

If C is kept constant, it can be seen that the theoretical effect on the amount of water in a single cell caused by a change of pH, temperature, or oxygen tension will be governed by the extremely simple relation:

$$\frac{W_1}{W_2} = \frac{2R+1-F_1}{2R+1-F_2}, \tag{10}$$

where W_1 and W_2 are the amounts of water in the cell and F_1 and F_2 are the amounts of base bound by a unit amount of hemoglobin under any two chosen conditions, R as defined above representing the ratio of base to hemoglobin within a single cell or any given quantity of cells. If, on the other hand, F is kept constant and C is varied, we have the usual osmotic equation,

$$\frac{W_1}{W_2} = \frac{C_2}{C_1}. \tag{11}$$

(F is actually not entirely independent of C, but for the small concentration differences here involved it may be considered to be.)

VII

It is now possible to determine whether or not the observed effects on osmotic hemolysis of the factors pH, temperature, and oxygen tension are of the order of magnitude of those predicted by the simple theory developed in the preceding section. A convenient method for

making the comparison is to convert equation (9) into the following form:

$$\frac{W_1}{W_2} = \frac{2R + 1 - F_1}{2R + 1 - F_2} \cdot \frac{C_2}{C_1}. \qquad (12)$$

C and F have here both been allowed to vary simultaneously, it being assumed that these variables are independent of one another, which under the conditions in question is approximately true.

Two observed points on a line of equal hemolysis are now chosen. By the osmotic theory equal percentages of hemolysis indicate the entrance into the cells of equal quantities of water, *i.e.*, under these conditions $W_1 = W_2$, and the resulting value of the right-hand side of equation (12) should be unity. If, therefore, on substituting in equation (12) the appropriate numerical values of C and F for the two chosen points together with the proper value of R for the blood in question, the expression becomes equal to, or nearly equal to one, the observed results may be said to be in agreement with the theory. Any considerable departure from this value, on the other hand, will indicate inadequacy of the theory.

Concerning the values of C there is no difficulty. The necessary values of R and F, however, cannot at present be obtained with the same degree of certainty, since the work here reported was done on the blood of two species, the ox and the cat, for which exact data concerning these quantities are apparently not yet available. Indeed, even if such data had been published, it is not likely that different animals of the same species would fail to show some individual differences. In view of the fact, however, that various simplifying assumptions have been made in the derivation of the equations, which at best are only approximate, and the further fact that, as far as is known, the bloods of different mammals resemble one another fairly closely, it would seem to be permissible to take from the literature such data as are at present available, expecting no more than that the calculated and observed results may perhaps prove to be of the same order of magnitude.

With regard to R, in the absence of any more definite information concerning the bloods actually used, we may take as a plausible value that given for the horse by equation (15) of Van Slyke, Wu and McLean (1923), namely, 6.0. This value will be used in all of our calculations.

Before the magnitude of the theoretical pH effects can be estimated, it must be noted that the pH values used in the calculations of the base-binding power of hemoglobin are those of the interior of the cell, while

those actually observed in the hemolysis experiments are those of the solution. The relation between the two by the Donnan principle is:

$$\text{pH cell} - \text{pH solution} = \log r,$$

where r can be obtained by means of equation (6), preferably first converted to the simpler form:

$$r = 1 - \frac{1 + F}{2R + 1 - F}.$$

To avoid the mathematical difficulties involved in attempting to determine pH cell from pH solution, it is convenient to work in the opposite direction, assuming a number of values of pH cell and calculating the corresponding values of pH solution. These latter values may then be plotted and a smooth curve drawn through them from which the desired values of pH cell may readily be obtained for any observed value of pH solution. In the calculations which follow this has been done.

Fairly extensive data for the calculation of pH effects are available for the blood of the horse at 38° C. Temporarily disregarding the difference in temperature, we may use for the calculation of F the equation given on page 152 of the paper by Hastings, Van Slyke, Neill, Heidelberger and Harington (1924) with the substitution of the necessary constants from Table XXIII for horse hemoglobin with a cation concentration of 145 mM.

Referring now to Fig. 3, we may choose any two points such as A and B which represent the same degree of hemolysis, for example, 75 per cent at pH 8.0 and pH 7.0 respectively. These particular points have been selected in order to keep within the range actually studied by Warburg (1922) and by Van Slyke, Wu and McLean (1923) and at the same time to take advantage of the region where our method of measuring hemolysis (Jacobs, 1930) is most accurate. The concentrations represented are 0.107 M and 0.079 M respectively. Remembering that the values of cell pH corresponding to solution values of 7.0 and 8.0 are 6.92 and 7.75, and assuming complete oxygenation of the hemoglobin at the tension of room air, we obtain by calculation F 7.0 = 0.97 and F 8.0 = 3.34. Substituting these various values, together with that of R in equation (12) we have finally

$$\frac{W_A}{W_B} = \frac{12 + 1 - 3.34}{12 + 1 - 0.97} \cdot \frac{0.107}{0.079} = 1.09.$$

The agreement between observation and theory is therefore seen to be fairly close. It may be noted that while the closeness of this

agreement will vary somewhat with the positions of the selected points, A and B, owing perhaps to the influence of unknown factors of secondary importance, the points actually chosen are by no means the most favorable that could have been selected for the theory. Furthermore, over a considerable range of pH and concentration, the observed and predicted results are at least of the same order of magnitude. This, under the circumstances, is all that reasonably can be expected.

The calculation just made involves a temperature differing considerably from the one at which the observations were made. It is not likely, however, if direct observations on the value of F at 20° C. were available, that their use would greatly change the ratio in question. According to Stadie and Martin (1924), the effect of temperature on the base-binding power of hemoglobin is exerted not upon the buffer value of the hemoglobin but only upon its isoelectric point. In other words, in the equation for reduced hemoglobin

$$[BHb] = \beta_R[Hb] \quad (\mathrm{pH} - \mathrm{pI});$$

a change of temperature causes a change in pI but not in β_R. Conditions for oxygenated hemoglobin are similar, though there is a very slight departure within the range in question from a simple linear relationship. Under these circumstances, in correcting our calculations so as to make them apply to 20° C., both numerator and denominator of the fraction:

$$\frac{2R + 1 - F_1}{2R + 1 - F_2}$$

will be changed in such a way that the value of the fraction itself is little altered. In other words, it appears to be approximately though not exactly true that a given pH change produces the same relative volume change at different temperatures.

The question last considered anticipates to some extent the discussion of the effects upon hemolysis of temperature changes at constant pH. For data upon the base-binding powers of hemoglobin at different temperatures we have used Fig. 1 of the paper by Stadie and Martin to which reference has already been made. Since the temperatures are here 38° C. and 15° C. we have selected as our own points for comparison A and B in Fig. 4 representing these two temperatures and a degree of hemolysis of 60 per cent. This percentage is as near to the region of the greatest accuracy of our instrument as the rather small amounts of blood used in this experiment permitted us to go. The concentration values are 0.081 M and 0.092 M, respectively. Since the relation between the pH of the cell and that of the surrounding medium is almost the same at a solution pH of 7.4,

which is that which we employed, and a blood pH of the same value, we have taken our figures directly from the graphs of Stadie and Martin without further calculations.

Considering first the figures for reduced blood, which are based upon actual observations, we find $F\ 38° = 2.6$ and $F\ 15° = 1.0$. Substituting in equation (12), we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 2.6}{12 + 1 - 1.0} \cdot \frac{0.092}{0.081} = 0.98.$$

If, instead of the observed figures for reduced blood, we take the calculated ones for oxygenated blood, we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 3.2}{12 + 1 - 1.6} \cdot \frac{0.092}{0.081} = 0.98.$$

Finally, we may use the theoretical effect upon pI calculated by Stadie and Martin from the heat of ionization of hemoglobin. They estimate that a change from 38° to 20° causes a change of pI for reduced blood of approximately 0.3 in the alkaline direction. Taking, therefore, values of pI of 6.8 and 7.1 for the two temperatures in question, and one of 2.8 for β_R we have for F, *i.e.*, for $[BHb]\ [Hb]$, $F\ 38° = 1.68$ and $F\ 20° = 0.84$. The concentrations for 60 per cent hemolysis for these two temperatures taken from our own data are 0.081 M and 0.089 M, respectively. Substituting these values as before in equation (12) we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 1.68}{12 + 1 - 0.84} \cdot \frac{0.089}{0.081} = 1.02.$$

This calculation, strictly speaking, applies only to reduced hemoglobin, but the results would not be very different under conditions of partial or complete oxygenation.

Finally, the factor of oxygenation at constant temperature and pH may be considered. Our own results upon hemolysis indicate a comparatively small osmotic effect of this factor. For the calculated effect at 38° the equation of Hastings, Van Slyke, Neill, Heidelberger and Harington (1924) already referred to may be used. As mentioned above, the difference in temperature in the two cases, while unfortunate, may be expected to have only a minor effect on the calculated result. At the pH of our experiment, namely 7.36, corresponding to an intracellular pH of 7.23 we find $F_o = 1.90$ and $F_R = 1.22$. For points A and B in Fig. 5, which are typical, the concentrations are 0.0862 M and 0.0875 M. Substituting these various values in equation (12) we obtain:

$$\frac{W_A}{W} = \frac{12 + 1 - 1.90}{12 + 1 - 1.22} \cdot \frac{0.0875}{0.0862} = 0.96.$$

Otherwise expressed, the value of the water ratio W_0/W_R calculated from the theoretical base-binding power of the hemoglobin is 0.94; that calculated from the observed equivalent concentration difference is 0.98. These values are of the same order of magnitude and both suggest the likelihood of much smaller errors in experimental work from neglect of the factor of oxygenation than of either of the others considered.

From the calculations which have been given concerning the theoretical osmotic effects of changes of pH, temperature and oxygenation, it is apparent that the predicted results are at least of the order of magnitude of those actually observed. The agreement is better in some parts of the range of the variables considered than in others and is nowhere perfect, as might be expected considering the various simplifying assumptions made in the derivation of our equations, and the necessity for using data obtained for other species of animals under dissimilar conditions. The existence of factors other than the purely osmotic ones is by no means excluded by our experiments; indeed it appears to be likely. We believe, however, that such factors are probably of secondary importance and that until the possibilities of the osmotic principles here discussed have been exhausted by calculations involving the use of more accurate data than are now available it will be unprofitable to indulge in speculations concerning little-understood "colloidal properties" of the cell and its constituents.

VIII

It has been shown that the influence upon hemolysis of the factors pH, temperature and oxygen can be at least largely accounted for by the osmotic effects which they produce through changes in the base-binding power of the hemoglobin. The question arises why the erythrocyte appears to stand alone in its sensitiveness to such factors. Hemoglobin is not a unique substance in its ability to bind base, nor in the effect upon its base-binding power of at least the factors pH and temperature. The proteins of all cells share with it these properties and yet within physiological limits other cells appear to be only slightly affected by the factors in question. Indeed, in the case of pH effects, Lucké and McCutcheon (1926) over a very wide range of reactions were unable to obtain unmistakable evidence of volume changes in the case of uninjured cells.

It is perhaps not possible in the present state of our knowledge of cells other than the erythrocyte to give a complete explanation of these differences, but attention may be called to a number of significant facts.

In the first place, even if a cell such as the *Arbacia* egg were freely permeable to anions and not to cations as is the erythrocyte—which is probably not the case—equation (10) would suggest a reason for a lesser liability of the former type of cell to volume changes. Other things being equal, the larger the value of R in this equation the less will be the effect upon the ratio W_1/W_2 of a given change in F. Now the value of R for the *Arbacia* egg is probably several times as large as that for the erythrocyte. We have little information about the concentration of its cell proteins as compared with that of hemoglobin in the erythrocyte, but it is almost certainly lower; the concentration of base, on the other hand, must be, roughly, three times as great, since the isotonic NaCl solution for the *Arbacia* egg is approximately M/2 as compared with M/6 for the erythrocyte. A value of R five times as great for the *Arbacia* egg as for the erythrocyte would therefore not be beyond the bounds of probability. Furthermore, the change in F with change of pH depends upon the buffer value of the protein in question as defined by Van Slyke (1922). Now the buffer value of hemoglobin is approximately three times as high as that of some of the commonest proteins such as albumins and globulins (Hastings, Van Slyke, Neill, Heidelberger and Harington, 1924). The absolute values of F, of course, are also of importance, but they cannot as yet be compared very accurately for the two types of cells. In any case, other things being equal, the high buffer value of hemoglobin tends to favor the production of large osmotic effects by pH changes.

In the second place, it seems almost certain that external changes in pH have far less effect upon the internal pH of a cell such as the *Arbacia* egg than of the erythrocyte. In the latter the simple Donnan principle: $\mathrm{pH}_{\mathrm{internal}} - \mathrm{pH}_{\mathrm{external}} = \log r$, governs the relation between internal and external reaction, however the latter may be produced. At ordinary reactions the difference is not usually greater than 0.2 pH units. On the other hand, in the eggs of *Asterias* and *Echinarachnius*, which are probably very similar to that of *Arbacia*, the internal pH is not only normally very different from that of the surrounding sea water, *e.g.*, 6.8 as compared with 8.1 (Chambers, 1928), but is relatively independent of the pH of the latter. Only freely penetrating acids, such as carbonic, butyric, etc., or freely penetrating alkalies, such as ammonia, are able, without injury to the cell, to cause visible changes in the appearance of intracellular indicators or to affect the cleavage process in a decided manner. According to Chambers (1928), even acids and alkalies of the naturally penetrating type in moderate quantities do not noticeably change the reaction of uninjured protoplasm but affect only certain cell inclusions. On the whole, therefore, the opportunities for

producing intracellular pH changes which alone could cause the type of changes described by equation (10) are very slight as compared with those existing in the case of the erythrocyte, and it is not surprising that the latter cell responds to such changes in a more or less unique manner.

Less is known about the effects of temperature than of pH on cells other than the erythrocyte. Obviously, however, the situation is very much more complicated in cells with a high rate of metabolism and a great variety of physiological activities than in the relatively simple erythrocyte. It would be expected, therefore, that in ordinary cells the effects predictable from equation (10) would be modified and obscured by others of a different nature. As far as it is legitimate to apply this equation, however, the remarks already made about the magnitudes of R and of F would hold in this case also. As for oxygen, the reasons for the unique behavior of the erythrocyte are so obvious that they require no special discussion.

IX

It appears from the foregoing discussion that the erythrocyte is a more or less unique type of cell. Its nature is such that apparently insignificant changes in environmental factors such as pH and temperature produce within it osmotic effects of a magnitude sufficiently great to be absolutely fatal to the securing of reproducible results by a method as sensitive as that of hemolysis.

It is now possible to appreciate the difficulties that have attended all work upon osmotic and perhaps, to a lesser extent, other types of hemolysis where the factors in question have been neither measured nor controlled. If it be true, as appears to be the case, that pH changes of 0.01 pH unit and temperature changes of 0.5° C. can produce measurable osmotic effects, what is to be said of the numerous papers which have been published upon the "fragility" of the erythrocytes under almost all conceivable normal and pathological conditions, in which in the absence of any information whatever concerning these factors an uncertainty of as much as 2.0 pH units and 10° C. or more may exist, and in which, in addition, there is frequently no assurance that the experiments have been sufficiently long continued to secure approximate equilibrium? It is disheartening to be forced to believe that a large part of the work in this much cultivated field is of very doubtful value, but there seems to be no escape from such a conclusion. The erythrocyte being what it is, attempts to use it for comparative osmotic studies without controlling especially the factors pH and temperature, and to

a lesser extent oxygen tension, are scientifically in the same category with attempts to study volumes of gases without accurate regulation of temperature and pressure.

In conclusion, a point mentioned in the first paper of this series may again be emphasized. The erythrocyte is not in itself a highly unreliable and capricious form of material, as it is frequently believed to be. Such capriciousness as it may appear to possess is merely a reflection of the carelessness of the experimenter or of his lack of understanding of its true physiological nature. When used uncritically merely as so much "material" for experiments in the field of General Physiology, the erythrocyte is likely to be a source of much vexation to the experimenter. When treated in a manner appropriate to a highly specialized cell with unique functions and physiological properties, concerning which more exact information is already available than is the case with perhaps any other single type of cell, it is capable of yielding results of an extremely satisfactory character.

Summary

1. By the method of osmotic hemolysis an attempt has been made to evaluate the indirect osmotic effects upon mammalian erythrocytes of changes in the pH, temperature, and oxygen tension of the surrounding medium.

2. The observed effects of these three factors, within the range considered, are of the order of magnitude of those predicted by the equation:

$$\frac{W_1}{W_2} = \frac{2R + 1 - F_1}{2R + 1 - F_2} \cdot \frac{C_2}{C_1},$$

where W_1 and W_2 are the amounts of water contained in an erythrocyte under two given conditions, F_1 and F_2 are the amounts of base bound by one equivalent of hemoglobin under the same conditions, C_1 and C_2 are the concentrations of the solutions in question and R is the ratio of base to hemoglobin within the cell. It is probable that the effects of the factors studied are primarily osmotic in nature, though smaller effects of a different sort are by no means excluded.

3. Certain differences between the osmotic behavior of the erythrocyte and that of other cells are discussed.

4. It is shown that pH changes of as little as 0.01 pH unit and temperature changes of as little as 0.5° C. may have a measurable effect upon the observed degree of hemolysis. It follows, therefore, that "fragility" tests and other osmotic studies upon erythrocytes in which these factors are not properly controlled are of little value.

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